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Review Article

DNA Amplification Techniques for the Detection of *Toxoplasma gondii* Tissue Cysts in Meat Producing Animals: A Narrative Review Article

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Abstract

Background: *Toxoplasma gondii* is an intracellular parasite, which infects one-third population of world. Humans and animals acquire infection by ingesting oocytes from feces of cats or by meat of other animals having cysts that may lead to congenital, ocular or cephalic toxoplasmosis. Either it is important to detect *T. gondii* from meat of food animals from retail shops or directly at slaughterhouses, which is meant for export.

Methods: The current research was done without time limitation using such terms as follows: "*Toxoplasma gondii*", "Meat", "Tissue cyst", "PCR", "LAMP", "Screening" and "Immunological assay" alone or in combination, in English language. The used electronic databases for searching included as follows: Pub-Med, Scopus, Google Scholar, Web of Science and Science Direct. The searches were limited to the published papers to English language.

Results: Sensitivity of different molecular techniques for diagnosis of *Toxoplasma* is real-time PCR > LAMP > conventional PCR. In addition to these DNA analysis tools, bioassay in mice and cats is considered as "gold standard" to detect *T. gondii*.

Conclusion: This review article will help the readers for grasping advantages and limitations of different diagnostic tools for screening meat samples for *T. gondii*. This review also makes bibliography about the type of meat sample to be processed for diagnosis and different primers or sequences to be targeted for *T. gondii* by number of researches for its detection from meat or tissue sample using DNA amplification techniques.

Introduction

Toxoplasma gondii is a ubiquitous obligate intracellular protozoan parasite, which belongs to phylum Apicomplexa (1). About one third of global population is infected with toxoplasmosis (2). It infects all warm-blooded animals including humans, birds, animals and marine mammals with worldwide distribution (3-5). Felines are the only final or definitive hosts for *T. gondii* with human and all other warm-blooded animals as intermediate hosts (4). Its life cycle is complicated with two infection mechanisms, one is by ingestion of oocysts produced by coccidian development in felines and the second is by utilizing the tissue cysts present in those intermediate hosts, which are chronically infected (6). As cat is the final host for *T. gondii,* thus, prevalence in cat is more important especially in stray cats (7). Cats can shed oocytes via feces in the environment, which are taken up by intermediate hosts orally, and tissue cysts are destined in skeletal muscle and brain tissue of these intermediate hosts. Man and animals are infected mainly by ingestion of raw or undercooked infected meat with tissue cysts, contaminated food or water with fecal oocysts and congenitally through trans-placental crossing of tachyzoites in pregnant subjects. Oral route is the main route out of these three infectious routes. About 30%-63% toxoplasma infection in human beings occurs by consumption of undercooked meat while infection from contaminated soil is about 6-17% (8-10).

Studies showed about $1/5^{th}$ meat consuming animals carrying *T. gondii* (11). As the infection is asymptomatic, it can be critical for congenitally infected and immunocompromised patients (12). For detection of *T. gondii* from meat samples, direct or indirect method can be used. New strategies for prevention of *T. gondii* transmission to humans should be introduced (13). So far, among different diagnostic techniques, PCR is the most specific diagnostic tool for characterization of toxoplasmosis than other techniques like serological, histological and tissue culturing (14).

This review article will compare DNA amplification techniques with serological techniques.

Methods

Method of Research and databases

The current research was done without time limitation using such terms as follows: "*Toxoplasma gondii*", "Meat", "Tissue cyst", "PCR", "LAMP", "Screening" and "Immunological assay" alone or in combination, in English language. The used electronic databases for searching included as follows: PubMed, Scopus, Google Scholar, Web of Science and Science Direct. The searches were limited to the published papers to English language.

Data collection and analysis

We extensively searched all above-mentioned databases. The bibliographic references were carefully screened to exclude the duplicates and non-related studies. Overall, 13 papers were selected. The following data were collected from the papers: first author, year of publication, geographical region of study, type of sample, type of animal and technique used to screen the samples. Finally, the extracted data were recorded in Table 1.

Results and Discussion

Conventional PCR

Molecular diagnosis like PCR is becoming new trend in diagnosis of parasites because of limitations in conventional diagnostic methods like simple microscopy due to resemblance in their morphology (15). Several PCR assays have been introduced to detect *T. gondii* by targeting B1 repetitive gene, rDNA, P30 gene and internal transcribe spacer (ITS-1).

Table 1: Type of tissue samples used for detection of *T. gondii*

Sample type	Area of Study	Animal	Type of sample	Reference
Cured meat samples	South West London	-	Dried, semi-dried and fermented sausages, and cured hams	(37)
Brain, Cardiac Muscles and Skeletal Muscles	Scotland, UK	Sheep and cattle	Experimentally infected sheep and cattle	(36)
Food samples	UK	Pork, Lamb and Beef	Commercial Food	(38)
Lung, Diaphragm, Brain, Lymph Node	India	Goats	Experimental animals	(39)
Heart, Tongue and Loin or center cut chops	Massachusetts	Swine	Naturally and Experimentally infected pork's meat	(40)
Tongue + Diaphragm	Erechim, Southern Brazil	Swine	Slaughtered meat	(41)
Diaphragm	Switzerland	Pig, Cattle, Sheep	Slaughtered meat	(42)
Boneless Cuts of Muscle tissue	China	Pork	Retail meat Stores	(34)
Hilar Lymph Node	China	Pork	Slaughtered animal	(43)
Heart Tissue	Tunis	Ewe	Slaughtered animals	(44)
Heart, Brain Liver	Iran	Chicken	Slaughtered animal	(45)
Brain, Neck Muscle and Tongue	Iran	Turkey	Slaughtered animal	(5)

For the preliminary attempt, PCR was applied for the detection of *T. gondii* by targeting B1 repetitive gene and found very useful method in diagnosis (16). PCR is much more precious in diagnosis of cases that showed Toxoplasma parasitemia while Tox-IgG based ELISA can determine the toxoplasma specific antibodies but it is unable to verify the acute toxoplasmosis (17). In bioassay of mice, capture ELISA, immunoblotting and PCR were compared after inoculation of tachyzoites. The results showed that PCR was the most sensitive assay as compared to other techniques as PCR detected *T. gondii* after 18 hours of post-infection while other techniques detected after 1 day of post-infection (18). Although conventional PCR is sensitive method for detection of *T. gondii* but it has a few limitations for the detection of *T. gondii* from the tissue cyst of animal. As DNA extraction requires a small amount (in milligrams) of tissue for DNA extraction while there are chances that the tissue cysts may not present in that part. So it will be difficult to detect *T. gondii* from tissue samples using PCR. Moreover, the conventional PCR cannot quantify the PCR product thus it cannot notify the level of infection but Real-time PCR can quantify PCR products. In case of conventional PCR, primers may bind at non-specific sites so amplification may likely be of undesirable template DNA sequence. However, in nested PCR, nonspecific binding of primers on template DNA can be prevented. As PCR is highly sensitive, there are many chances of contamination from even a single amplified DNA trace if found, may give false positive results.

Real-time PCR

Real-time PCR-based assay quantifies PCR amplified product with fluorescent technology. This assay quantifies the resultants PCR product during each cycle of the PCR amplification also the proportion of starting and end numbers of gene to be amplified (19). Sensitivity of Real-time PCR was 95.5% with probe targeting the repetitive B1 gene of *T. gondii* to detect and quantify from human peripheral blood (20). The Real-time PCR assay using FRET protocol by targeting a 529 bp repeat region is considered as more sensitive and specific for *T. gondii* from immunocompromised patients and pregnant women as compared to Real-time PCR based on TaqMan protocol, which targeted 18S RNA gene, and to nested PCR which targeted B1 gene of *T. gondii* (21). Real-

time PCR by targeting B1 gene was considered as more accurate and desirable assay as compared to nested and conventional PCR (22). As bioassay is gold standard for detection of *T. gondii* but it is not good for ethical point of view. A new technique had been studied for detection and quantification of *T. gondii* as an alternate of bioassay in laboratory animals. The DNA was extracted from the large amount of tissue sample by magnetic capture and Real-time PCR was done by targeting the B1 gene sequence (23). Advantages of Real-time PCR over conventional and nested PCRs are that it is easy to use, minimize the contamination risks, produces results quicker and in a quantitative way (21, 24).

Loop-mediated Isothermal Amplification (LAMP)

LAMP is a distinctive molecular diagnostic technique, which amplifies desired DNA fragment using isothermal conditions by use of four primers that identify six regions on DNA fragment to be amplified (25). Loop-mediated isothermal amplification (LAMP) method amplifies DNA with high specificity, sensitivity and rapidity (26). LAMP is more sensitive than conventional PCR but not from real-time PCR (26, 27). In comparison between quantitative PCR and LAMP, Q-PCR was found more sensitive as compared to LAMP (28). LAMP detected *T. gondii* from blood of experimental mice infected with tachyzoites of RH strain by targeting B1 gene and it was found sensitive, simple and cost-effective tool for diagnosis (29). LAMP is an attractive diagnostic assay in field conditions where sophisticated and expensive tools are not available (28). LAMP assay is probably a substitute tool for detection of *T. gondii* in the field condition but real-time PCR assay is the most sensitive diagnostic tool for its detection (27).

Potential samples of T. gondii tissue cysts

Not all types of meat are responsible for toxoplasmosis in humans; it mainly depends on prevalence of *T. gondii* in animals and the way or type of meat consuming behavior of human being (30-32). For example, frozen meat of animals is a potent source of toxoplasmosis when is consumed such as of buffalo in Turkey (33) or pork meat in china (34). However, refrigeration of meat kills the parasites but for clients, the major risk factor is purchasing and then consuming meat from retail shops as such. Viable organism of *T. gondii* was isolated from pork in retail shops (35). The type of tissue sample taken from the animal has been enlisted in Table 1.

Cattle are more resistant to *T. gondii* infection as compared to ovine. It is the most important cause of congenital problems in sheep as compared to all other warm-blooded animal species. As sheep remains persistently contaminated to *T. gondii* infection for whole life, its undercooked meat is a major source of infection in humans. However, in cattle, very few tissue cysts develop which may not persist for whole life, so they are less life threatening to humans (36).

Utility of different genes for detection T. gondii

In Switzerland, meat samples from pigs, ovine and bovine were detected as positive for *T. gondii* infection using B1 gene by PCR. In this study, calves showed more prevalence as compared to heifers, bull, cow, pig and sheep (42).

The pathogen can be detected in human patients with acute myelogenous leukemia or fetuses with hydrocephalus and chorioretinitis, by targeting BI gene using forward and reverse primers as 5'-TTGCATAGGTTGCAGTCACT-3' (positions: 694 to 714) and 5'-TCTTTAAAGCGTTCGTGGTC-3' (positions: 868 to 888) respectively. In these cases by using brain tissue, PCR showed much better results as compared to tissue culturing and IFT after tissue inoculation in mouse (46).

S48 strain of *T. gondii* was analyzed for genomic DNA of organism by amplification of B1 and P30 genes of toxoplasma by PCR

from efferent lymph and peripheral blood, obtained from artificially infected sheep with *T. gondii*. The efficacy of both genes for detection of organism by amplification method was further analyzed by inoculation of sample in mouse. Results showed that PCR of B1 gene was more sensitive as compared to P30. P30 gene after PCR gave more false negative results than B1 gene, but both B1 and P30 PCR detected organism's DNA from lymph node of experimental sheep after 12 days of infection but not in any other tissue. This study showed that PCR is the most specific, sensitive and reliable research diagnostic tool for detection of toxoplasmosis than histological detection and tissue culturing (14). The use of different primers and genes for molecular detection of *T. gondii* has been highlighted in table 2.

Bioassay

Screening of positive slaughtered meat can also be done by bioassay in mice and cats. Bioassay in cats is considered as "gold standard" for detection of *T. gondii* (40, 47) because a large amount of tissue samples can be fed to cats and tissue samples with low load of bradyzoites can induce the shedding of oocysts in feces of cats (48, 49). Positive suspension of *T. gondii* was given orally to the experimental animals on daily basis and after 6 and 12 months, animals were slaughtered.

Table 2: Most common genes and primers used for detection of *T. gondii* from meat samples

Gene Target	Forward primer sequence	Reverse primer sequence	Fragment size	Annealing temperature	Study
microsatellite sequence	5'-CGCTGCAGGGAGGAAGAC-GAAAGTTGAG	5' -AGCGCTGCAGACACAGTG-CATCTGGATT	533 bp	56 °C	(41)
P30	5'-TTGCCGCGCCCACACTGATG	5'-CGCGACACAAGCTGCGA-TAG	914 bp	60 °C	(37)
B1	5'-GGAGGACTGGCAAC-CTGGTGTCG	5'-TTGTTTCACCCGGAC-CGTTTAGCAG	2214 bp	60 °C	(42)
B1	B22: 5'-AACGGGCGAGTAGCAC-CTGAGGAGA	B23: 5'-TGGGTCTACGTCGATGG-CATGACAAC		57 °C	(44)
B1	5-GGAACTG-CATCCGTTCATGAG-3	5-TCTTTAAAGCGTTCGTGGTC-3		60 °C	(40) (39)
B1	5'-CCGTTGGTTCCGCCTCCTTC	5'-GCAAAACAGCGG-CAGCGTCT	432 bp	57 °C	(5)
B1	5'-CCGCCTCCTTCGTCCGTCGT	5'-GTGGGGGCGGAC-CTCTCTTG	213 bp	58 °C	(5)
ITS-1	5'-AGTTTAGGAA-GCAATCTGAAAGCACATC- 3'	5'-GATTTGCATTCAAGAA-GCGTGATAGTAT-3'			(45)

Then to detect *T. gondii*, tissue samples of brain, cardiac muscles and skeletal muscles were homogenized, incubated, filtered and then centrifugation at 1000g was injected in mice intraperitoneally. Mice that showed symptoms of disease like dehydration, emaciation and tottering gait were culled for further examination and diagnosis. Microscopic examination of tachyzoites using peritoneal exudates and ELISA was performed for the detection of IgG specific for *T. gondii* using blood samples (36).

In experimentally infected goats, only female showed signs of illness after being infected.

The signs of illness were dull, off-feed and rhinitis with mucopurulent nasal discharge with abortion and ultimately death of experimental animal. Postmortem examination exposed severe metritis with edematous and enlarged lymph node. Samples aspirated from lymph node after processed with lysis buffer showed specific amplified product of 194 bp as compared to direct amplification of samples. PCR amplified product is more prominent in case of lymph node samples as compared to muscle homogenate (39).

In another study, brain tissues from slaughtered turkeys were injected intraperitoneally after homogenization in mice and observed daily for two months. After that, blood and brain samples from mice were taken for serological and molecular diagnosis of *T. gondii* (5). Halos et al., bioassayed whole heart in mice after it was mixed thoroughly and incubated at 37°C for 2.5 hours along with trypsin. Then after filtration, centrifugation and washing, it was resuspended in saline solution that contained antibodies. This mixture was inoculated in mice and after four weeks, their serum was collected and tested by MAT for specific toxoplasma antibodies. Brains of these mice were used for detection of brain cysts by using PCR (11).

Although bioassay of tissue samples in experimental animals is a fair way for the detection of *T. gondii* from brain cysts of mice and from shedding oocysts from cat's feces but it cannot be used on large scale for the detection of antigen from meat or tissue samples. Because it is time consuming, expensive method and is not attractive ethically. However, it may also fail because of low or non-viability of parasite in tissue and part of tissue to be selected (50). PCR is the best alternative technique for the detection of *T. gondii* other than bioassay in live animals (23).

Advantage of PCR over serological techniques for detection of T. gondii

Different studies showed high sensitivity and specificity of different serological techniques for detection of *T. gondii*. Sensitivity and specificity of different serological techniques are summerized in table 3. In a comparative study between commercially available enzyme-linked immunosorbent assay (ELISA) and modified agglutination test (MAT), *T. gondii* specific antibodies were detected from naturally infected pigs by bioassay in cats and from diaphragm and heart juices. Overall sensitivity was 88.6% and 85.7% for both ELISA and MAT respectively while specificity was 98% and 94.6% for ELISA and MAT respectively. These results showed ELISA has more sensitivity and specificity for detection of *T. gondii* as compared to MAT (47). But in a previous study in 1995, MAT was found as more sensitive and specific serological test as compared to all other serological techniques i.e. ELISA, LAT and IHAT (51). As shown in Table 3, sensitivity and specificity of ELISA is much better than other serological tests, but studies showed that by using protein of P30 gene as antigen, ELISA had given good results by detecting *Toxoplasma* specific antibodies from positive sera but it was unable to detect antibodies in field condition (47). When these techniques were compared with MAT, there were some drawbacks using MAT. The antibody detection needed whole antigen, more time was required for detection and difficult to interpret its results (47). Some studies showed low sensitivity and specificity of PCR, it was because small sample size, random distribution and low number of tissue cysts in tissue (40). Sensitivity and specificity for ELISA with PCR was 92.16% and 96.36% respectively while with MAT, it was 81.81% and 92.15% respectively but bioassay in mice showed only 27.36% samples positive for *T. gondii* as compared to PCR (45). However, serological techniques detect *T. gondii* infection qualitatively and quantitatively but it is difficult to differentiate the strain variation or genotype of the organism (52). The advantage of using immunological assays like MAT and ELISA etc. can be to perform the test on both live and dead animals and the antibodies are distributed evenly in the body fluids (42).

Table 3: Sensitivity and Specificity of different serological tests

Test	Sensitivity (%)	Specificity (%)	Reference
ELISA*	88.2–97.4	77.1–100	(56)
	72.9	85.9	(51)
	95.1	-	(57)
	88.6	98	(47)
	94	92	(58)
	93.1	98.8	(50)
	98.9	92.7	(50)
MAT**	82.9	90.3	(51)
	85.7	94.6	(47)
IHAT***	29.4	98.3	(51)
LAT****	45.9	96.9	(51)
IFAT*****	87.3	87	(50)
Western blotting	93.5	77.2	(50)
Dye Test Antibody	54.4	90.8	(51)

*ELISA: Enzyme Linked Immunosorbent Assay
**MAT: Modified Agglutination Test
***LAT: Latex Agglutination Test
****IHAT: Indirect Haemagglutination Test
*****IFAT: Immunoflourescent Antibody Test

Rather molecular techniques require tissue/meat sample for screening dead/slaughtered animal. The most important advantage by using PCR to classify the organism into different genotypes as genotyping is important to identify the potential of zoonosis (53-55).

Conclusion

As humans and animals especially immunocompromised patients and pregnant females, are at high risk due to consumption of raw and semi-cooked meat that contains cysts of *T. gondii*. It should be the priority to diagnose and screen out meat of slaughtered animals, which are positive for *T. gondii* before it reaches to the end user. Uptill now, PCR is the robust, sensitive and reliable molecular tool for characterization of *T. gondii*. Due to the large size of carcass, it is important for the researchers to select proper tissue for DNA extraction, which could be mostly diaphragm, heart, brain and muscle tissues, because they contain mostly the bradyzoite stage of *T. gondii*. B1 and P30 genes are the most targeted genes for the detection of *T. gondii* with different primers. They are resulting in different fragment sizes depending upon the type of primer used in PCR. Among these, B1 is more sensitive as compared to the P30 gene. Bioassay in cat is considered as "gold standard" and it is a reasonable assay for diagnosis of *T. gondii* but it has a few limitations as for its diagnosis; it is time consuming to pass oocysts in cat's feces or to develop brain cysts in mice.

On large scale or in field condition, it is impossible to commercialize the bioassay for diagnostic purpose to screen slaughtered meat or tissue because it is lengthy, expensive. Moreover, it has ethical and commercial issues because slaughtered meat is to be consumed within a few days after animals being slaughtered. Real-time PCR is the best alternate technique for the detection of *T. gondii* other than conventional PCR, LAMP, bioassay in live animals and serological techniques. This review article is helpful to present the bibliography regarding detection of *T. gondii* from indigenous or export meat as highlighting different type of samples and most commonly used primers for performing PCR. It is necessary to detect the organism from meat as early as possible after

slaughtering because of its tendency to infect humans.

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